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Activation of Cellular and Heterologous Promoters by the Human Herpesvirus 8 Replication and Transcription Activator

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The key regulator of the switch from latent to lytic replication of the human herpesvirus 8 (HHV-8; KSHV) is the replication and transcription activator (Rta). The ability of Rta to regulate cellular gene expression was examined by transient transfection into cells that were not infected with HHV-8. Rta induced some, but not all, NF-κB-responsive reporters through mechanisms that did not involve activation of classic forms of NF-κB. Furthermore, transfection of the NF-κB subunit Rel A inhibited the ability of Rta to transactivate some but not all reporters. For example, Rel A inhibited the ability of Rta to transactivate the IL-6 promoter, but only when sequences upstream of the NF-κB site were present. The ability of Rel A to inhibit Rta-mediated transactivation was not dependent on a functional NF-κB site within the promoter, suggesting an indirect mechanism for inhibition. These studies suggest that Rta expression during lytic reactivation of HHV-8 would lead to expression of some cellular genes, including IL-6, whereas activation of NF-κB could inhibit some responses to Rta.

Key Words: human herpesvirus 8; Kaposi's sarcoma herpesvirus; Rta; ORF 50; transcription; lytic reactivation; cellular promoters; NF-κB.

INTRODUCTION

Human herpesvirus 8 (HHV-8), also known as Kaposi's sarcoma-associated herpesvirus (KSHV), is etiologically associated with Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and an AIDS-associated plasmablastic variant of multicentric Castleman's disease (MCD) (Schulz, 2000). HHV-8 is a member of the gammaherpesvirus subfamily and bears significant homology to Epstein–Barr virus (EBV) and herpesvirus saimiri (Russo *et al.,* 1996).

Herpesviruses establish two modes of infection, latent and lytic, depending upon the cell type infected and other physiological parameters (Roizman, 1996; Wagner, 1991). Maintenance of episomal viral genomes, highly restricted gene expression, and a lack of progeny production characterize latency. During lytic, or productive infection, gene expression proceeds through a temporally regulated cascade leading to viral DNA replication and the production of progeny virions. A key HHV-8 regulator of the switch from a latent to lytic program is Rta (replication and transcription activator), also known as ORF 50 (Lukac *et al.,* 1998, 1999; Sun *et al.,* 1998), activator of replication and transcription (ART) (Wang *et al.,* 2001), and Lyta (Sakakibara *et al.,* 2001). Rta is encoded within a bicistronic transcript containing five exons: a small 5′ exon located upstream of open reading frame 49 (ORF 49), which includes the initial coding sequences; a second exon generated from ORF 50 encompasses the remaining coding sequence of Rta; and three additional exons corresponding to the KbZIP (ORF K8) transcript (Lukac *et al.,* 1999). The Rta protein is highly phosphorylated and contains multiple domains including amino-terminal basic and leucine zipper motifs and a carboxyl-terminal transactivation domain (Lukac *et al.,* 1999). Rta induces the expression of a number of HHV-8 promoters in both HHV-8-infected PEL cell lines and uninfected cell lines (Gradoville *et al.,* 2000; Lukac *et al.,* 1999; Sun *et al.,* 1998) and is sufficient to drive the lytic program to progeny virion production (Gradoville *et al.,* 2000). Additionally, expression of a dominant-negative Rta that lacks a transactivation domain blocks HHV-8 lytic reactivation induced by multiple stimuli (Lukac *et al.,* 1999).

Several studies have begun to elucidate the mechanisms involved in Rta-mediated gene induction. Sequences in the HHV-8 viral interferon regulatory factor-1 (vIRF-1) (Chen *et al.,* 2000), ORF 57 (Lukac *et al.,* 2001), Rta (Sakakibara *et al.,* 2001), and PAN (also known as nut-1 or T1.1) (Song *et al.,* 2001) promoters that are responsive to Rta have been identified. Recombinant Rta directly binds to a palindromic sequence in the ORF 57 and K-bZIP (ORF K8) promoters (Lukac *et al.,* 2001) and to a distinct sequence in the PAN promoter (Song *et al.,* 2001), yet these sequences are not present in some Rta-responsive promoters (Lukac *et al.,* 2001). Although recombinant Rta can bind to these sequences, Rta that is present in nuclear extracts from PELs fails to bind to

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these same sequences (Lukac *et al.,* 2001; Song *et al.,* 2001). Cellular transcription factors have also been shown to be involved in Rta-mediated transcription. For example, Oct 1 has been shown to be important for Rta-mediated transactivation of the Rta promoter. Rta also binds to the coactivator CREB-binding protein (CBP), leading to enhanced transactivation, whereas binding of histone deacetylase (HDAC) by Rta suppresses its ability to induce gene expression (Gwack *et al.,* 2001a).

In this study, we sought to characterize the consequences of Rta in the absence of HHV-8 infection on non-HHV-8 viral and cellular promoters. We demonstrate that (1) some but not all κB-containing promoters were activated by Rta; (2) Rta induction of NF-κB-responsive promoters was resistant to inhibition by IκBα and hence was distinct from induction by the NF-κB subunit Rel A; (3) transfected Rta resulted in interleukin-6 (IL-6) promoter activation and secretion of cellular IL-6; (4) multiple *cis* elements, including activating transcription factor (ATF), CAAT enhancer binding protein β (C/EBPβ), and nuclear factor κB (NF-κB) sites were not essential for Rta induction of the IL-6 promoter but contributed to responsiveness in some situations; (5) Rta induction of some, but not all, promoters was inhibited by cotransfection of Rel A; and (6) the ability of Rel A to inhibit Rta-mediated transactivation of the IL-6 promoter was not dependent on an intact NF-κB-binding site. These data suggest that induction of cellular genes by Rta may contribute to the pathogenesis of HHV-8-associated diseases and that in some situations NF-κB modulates Rta responsiveness.

RESULTS

Rta induces some but not all NF-κB-responsive promoters

Since Rta is a potent activator of HHV-8 genes and has been shown to interact with several cellular factors, we were interested in examining the ability of Rta to induce cellular or viral promoters in the absence of HHV-8-derived sequences. We first examined the effects of Rta on three heterologous promoter constructs (Fig. 1A): PRDII$_4$-CAT, which contains four tandem NF-κB responsive elements (PRD II) from the interferon-β promoter linked to −128 to +20 of the β-globin promoter (Fan and Maniatis, 1989), as well as PRDI$_4$-CAT and PRDIII$_6$-CAT, which have the κB elements replaced with tandem IRF-responsive elements from the interferon-β promoter linked to −128 to +20 of the β-globin promoter (Fan and Maniatis, 1989; Keller and Maniatis, 1988). Thus, these reporter constructs have identical transcription start sites and coding regions and differ only in the composition of the upstream PRD elements. PRDII$_4$-CAT was strongly activated by Rta, whereas PRDI$_4$-CAT or PRDIII$_6$-CAT were minimally responsive with induction that was comparable to that of induction of the β-globin promoter alone (Fig. 1B). These data indicate that the tandem PRDII elements were responsive to induction by Rta, whereas neither PRD I or PRD III were responsive.

To examine the mechanisms involved in the Rta responsiveness of the NF-κB-responsive construct PRDII$_4$-CAT, we compared the induction by Rta to induction by the NF-κB subunit Rel A. Rta induced PRDII$_4$-CAT to levels comparable to that of the NF-κB subunit Rel A (Fig. 1C). However, while the induction of PRDII$_4$-CAT by Rel A was strongly inhibited by cotransfection of IκBα, an inhibitor of NF-κB, Rta induction of PRDII$_4$-CAT was not affected by IκBα (Fig. 1C). Thus, the mechanisms involved in PRDII$_4$-CAT induction by Rta and transfected Rel A are at least in part distinct.

The ability of Rta to activate another NF-κB-containing construct, HIV(κB)$_4$-CAT, was then tested. HIV(κB)$_4$-CAT was constructed from the HIV LTR through duplication of tandem κB elements and deletion of some elements, including an Sp1 site (Kunsch *et al.,* 1992). Thus, this construct has multiple κB elements, similar to PRDII$_4$-CAT, but also contains additional HIV elements. Although the HIV κB sequences (5′-GGGGACTTTCC-3′) differ somewhat from the PRDII site (5′GGGAAATTCC-3′), both are responsive to the NF-κB subunit Rel A. While Rel A strongly induced HIV(κB)$_4$-CAT, Rta did not induce CAT activity from this construct (Fig. 2). Thus some, but not all, NF-κB-responsive promoters are inducible by Rta.

Rta induces the interleukin-6 promoter

To explore the effect of Rta on cellular promoters, we examined the ability of Rta to induce −435 IL6-CAT, a construct that contains −435 to +70 of the human IL-6 promoter (Fig. 3A). In 293T cells, cotransfection of Rta and −435 IL6-CAT resulted in a large induction of CAT activity (Fig. 3B). This induction was also observed using a nontagged Rta construct, pcDNA-FLc50, but not with mutant Rta lacking the carboxyl-terminal transactivation domain, pCMV-myc-nuc-50ΔSTAD (data not shown).

A number of *cis* elements involved in IL-6 promoter induction have been identified: AP-1 (−283 to −277), IRF-1 (−267 to −254), ATF (−163 to −158), C/EBPβ (−154 to −146; −83 to −75), Sp-1 (−126 to −101), as well as NF-κB sites (−75 to −63) (Fig. 3A) (Akira and Kishimoto, 1997; Faggioli *et al.,* 1997; Sanceau *et al.,* 1995). There are also numerous glucocorticoid response elements (GRE) (Ray *et al.,* 1990), a putative retinoblastoma control element (RCE) (Ray *et al.,* 1990; Takeshita *et al.,* 2000), and an RBPJκ-binding site (Kannabiran *et al.,* 1997; Palmieri *et al.,* 1999; Plaisance *et al.,* 1997) that function in negative regulation of the IL-6 promoter.

IL-6 promoter deletion constructs were used to identify regions of the IL-6 promoter important in Rta responsiveness. Deletion of the IL-6 promoter to −227, which deleted the AP-1 and IRF-1 sites, resulted in a 41% reduction in Rta-induced CAT activity compared to Rta induction of −435 IL-6 CAT (Fig. 3B). Deletion to −160, which

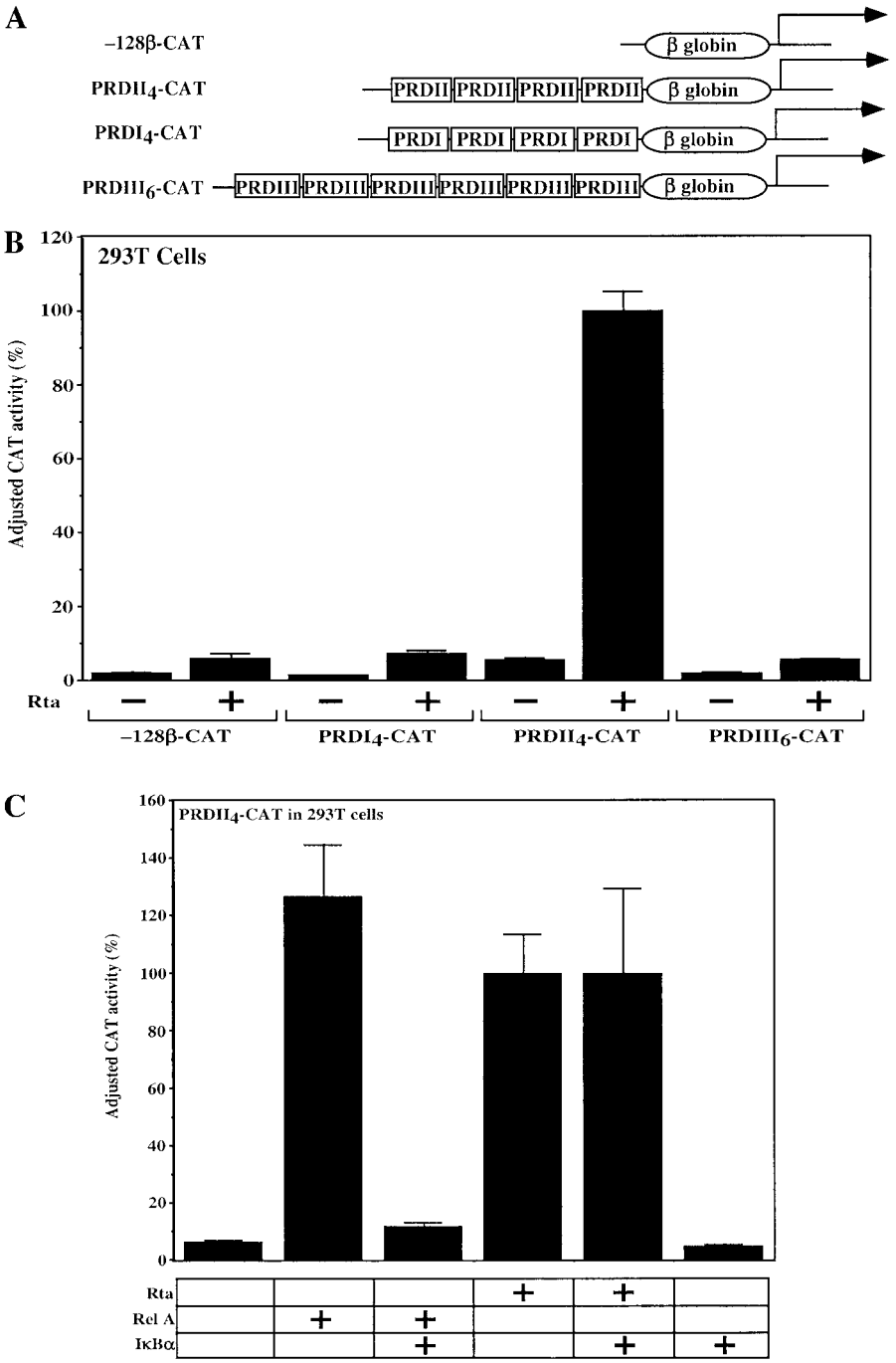

FIG. 1. Rta transactivates PRDII$_4$-CAT through a mechanism that is not inhibited by IκBα. (A) Diagram of reporter constructs. The −128β-CAT contains −128 to +20 of the β-globin promoter and is a common element for all constructs. The remaining constructs represent tandem copies of PRD I, II, and III elements from the IFNβ promoter linked to −128β CAT, as illustrated. (B) 293T cells were transfected with the indicated CAT reporter construct and pEF-LacZ along with vector (−) or pcDNA-FLAG-ORF 50 (Rta) (+). Seventy-two hours posttransfection, lysates were harvested and assayed for CAT and β-galactosidase (β-gal) activity. CAT activity was standardized to β-gal to correct for differences in transfection efficiencies. This representative graph depicts relative CAT activity and standard deviation of triplicate samples. Cpm/β-gal for all samples are compared to that of Rta-induction of PRDII$_4$-CAT, which is set at 100%. (C) PRDII$_4$-CAT and expression vectors for Rta (pcDNA-FLAG-ORF 50), the NF-κB subunit Rel A (pCMV-p65), or IκBα (pCMV-IκBα) were transfected into 293T cells as described under Materials and Methods, and CAT activity was measured at 72 h posttransfection. Cpm/β-gal for all samples are compared to that of Rta-induction of PRDII$_4$-CAT, which is set at 100%.

deletes the GRE and ATF sites, decreased Rta induction by 63%, whereas deletion to −130 IL-6 CAT, which removes the 5′C/EBPβ site as well as the other upstream elements, restored Rta responsiveness to levels that were comparable to that of −435 IL-6 CAT. Rta induction of −100 IL-6-CAT, which lacks the putative retinoblas-

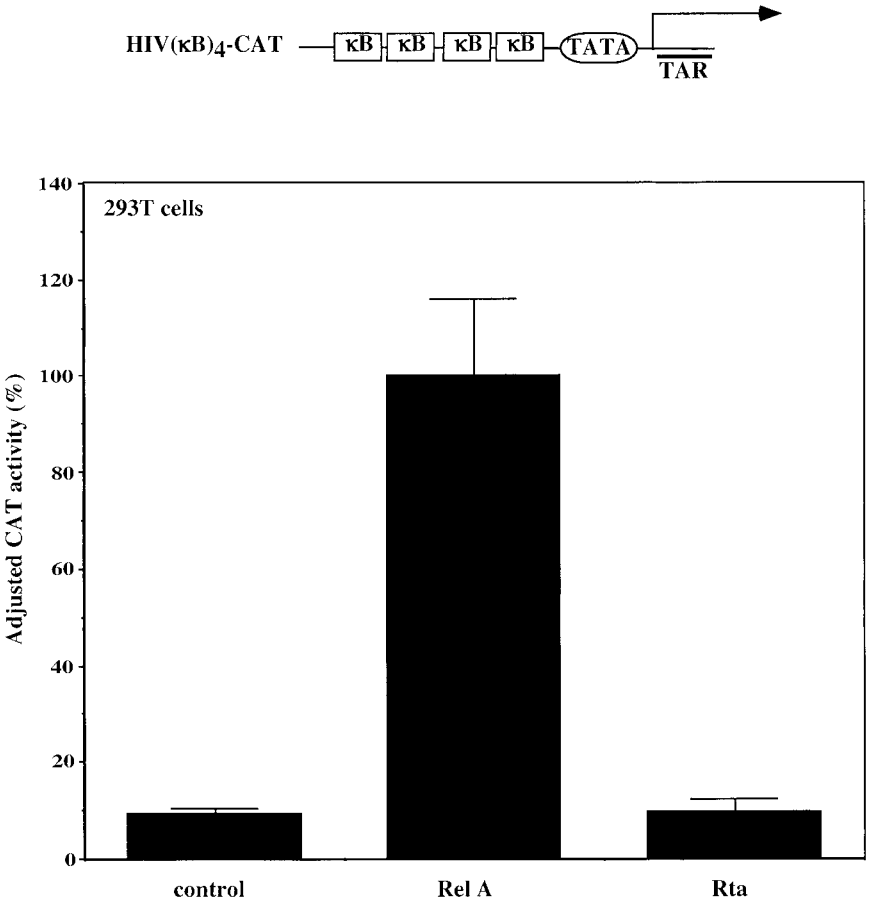

FIG. 2. Rta does not activate HIV(κB)$_4$-CAT. (A) Diagram of HIV(κB)$_4$-CAT. (B) 293T cells were transfected with HIV(κB)$_4$-CAT and pEF-LacZ along with control, pcDNA-FLAG-ORF 50 (Rta), or pCMV-p65 (Rel A). CAT activity is expressed as a percentage of Rel A-induced activity (defined as 100%). This representative graph depicts the mean ± standard deviation of triplicate samples.

toma control element and an Sp1 site, was 66% less than that of −435 IL-6 CAT, although significant levels of CAT activity were still induced. When the IL-6 promoter was truncated to −52, which deletes the 3′C/EBPβ and the NF-κB elements, Rta responsiveness of the promoter was abolished. Thus, sequences between −100 and −52 are critical for Rta induction of IL-6-CAT, whereas sequences upstream of −100 were not essential.

Changes in the level of promoter activity resulting from serial deletion of promoter elements suggested the presence of elements that both positively and negatively affected induction by Rta. The −130 IL-6 CAT construct that lacked the 5′C/EBPβ and ATF elements was as responsive to Rta as the −435 IL-6 CAT, indicating that neither element was required for Rta responsiveness. Deletion to −52 IL-6 CAT led to complete loss of responsiveness to Rta, suggesting that the NF-κB element might be required for Rta responsiveness, but other elements were also lost in this construct. Mutational analysis was therefore used to explore the role of specific sites in Rta responsiveness. The NF-κB, ATF, and 5′C/EBPβ sites have been shown to be important in induction of IL-6 by other inducers (Harcourt and Offermann, 2000; Hu *et al.,* 2000; Vanden Berghe *et al.,* 2000) and were examined for their role in Rta responsiveness. Rta was cotransfected with wild-type −435 IL6-CAT or with −435 IL6-CAT constructs containing mutations in the indicated sites (Fig. 4A), and CAT activity was assessed 72 h posttransfection. Mutation of either the ATF or the 5′ C/EBPβ sites in −435 IL6-CAT resulted in a modest decrease in induction of CAT activity by Rta, and mutations in both sites reduced but did not eliminate Rta responsiveness (Fig. 4B). Mutation of the NF-κB site in −435 IL6-CAT reduced but did not eliminate activity, and the reduction was less than the reduction that occurred when both the ATF and the C/EBPβ sites were mutated. Thus, none of these sites were required for Rta responsiveness, yet mutation of each of these sites decreased the level of induction.

The NF-κB subunit Rel A and Rta activate the IL-6 promoter in distinct ways and functionally interact through mechanisms involving promoter sequences upstream of the NF-κB site

The −100 IL-6 CAT construct that was responsive to Rta contains a NF-κB site but lacks multiple known

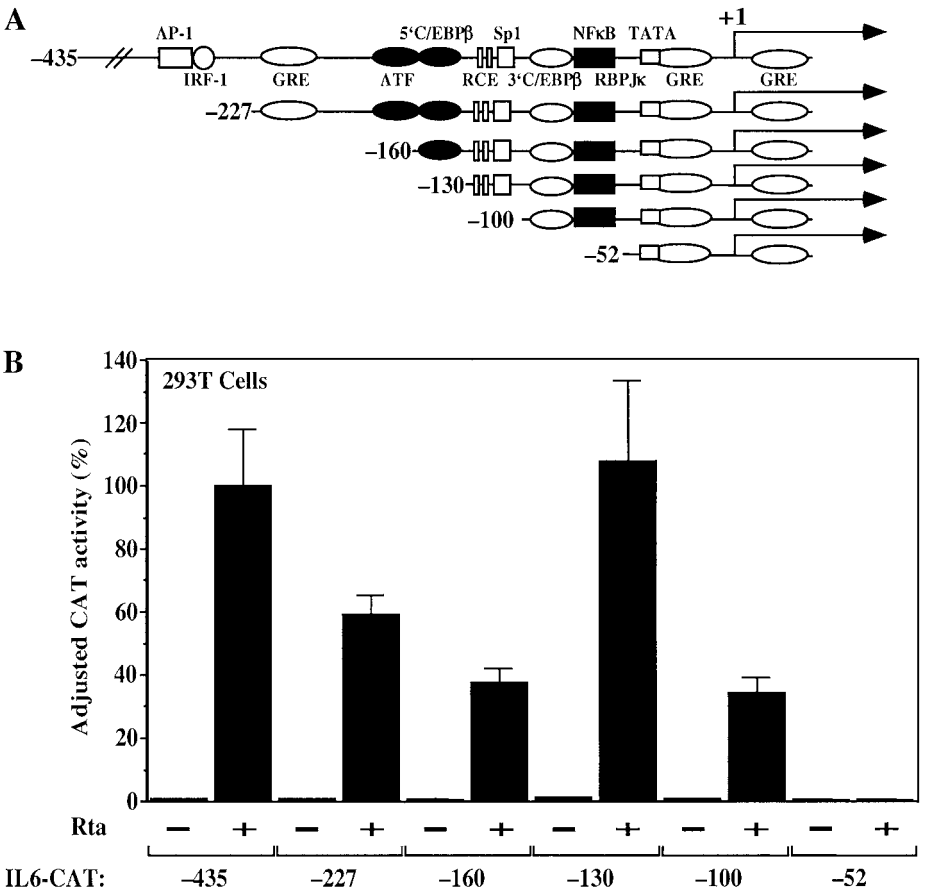

FIG. 3. Multiple upstream elements affect the level of interleukin 6 promoter-CAT activity in response to transfected Rta. (A) Diagram of the IL6-CAT truncation constructs. AP-1, activating protein-1; IRF-1, interferon regulatory factor-1; GRE, glucocorticoid response element; ATF, activating transcription factor; C/EBPβ, CAAT enhancer-binding protein; RCE, retinoblastoma control element; Sp1, stimulating protein 1; NF-κB, nuclear factor κB; TATA, TATA box. (B) Transient reporter assays with the indicated IL6-CAT constructs in the absence and presence of transfected Rta were performed in 293T cells as described in the legend to Fig. 1B. Rta induction of wild-type −435 IL6-CAT was defined as 100%.

positive regulatory elements, including the ATF and 5′ CEBP/β sites. To determine whether elimination of these upstream elements increased the need for an intact NF-κB site for Rta responsiveness, −100 IL6-CAT constructs containing either wild-type or mutated NF-κB site were cotransfected with Rta into 293T cells, and CAT activity was assessed 72 h posttransfection. Rta induced −100 IL6-CAT containing the intact NF-κB site, whereas mutation of the NF-κB completely abolished activation by Rta (Fig. 5A), indicating that the NF-κB site was necessary when upstream elements were missing.

We showed that Rta specifically induced the NF-κB-responsive construct PRDII$_4$-CAT, but that this induction was not inhibited by IκBα (Fig. 1C). This suggests that Rta and NF-κB may activate transcription in part through the same consensus element but through different mechanisms. To compare the induction of IL6-CAT by Rta and Rel A, we cotransfected IL6-CAT reporter constructs with Rta, Rel A, and IκBα, individually and in combination, into 293T cells. −100 IL6-CAT construct was responsive to either Rta or Rel A individually (Fig. 5B). Similar to induction of PRDII$_4$-CAT, IκBα had no effect on Rta induction of −100 IL6-CAT, whereas induction by Rel A was completely inhibited by IκBα. Cotransfection of Rta and Rel A led to levels of reporter activity that were comparable to that of Rta alone. Thus, Rta induction of −100 IL6-CAT was dependent upon the NF-κB sequence but was not mediated by a factor that is inhibitable by transfected IκBα.

When responsiveness to Rta and Rel A was compared using the −435 IL6-CAT reporter, Rta induced CAT activity, but Rel A was insufficient (Fig. 5C). Intriguingly, cotransfection of Rel A together with Rta greatly diminished Rta-mediated induction, whereas IκBα did not inhibit Rta induction of −435 IL6-CAT (Fig. 5C). Thus, functional interactions between Rta and Rel A were apparent when sequences between −435 and −100 were present (Fig. 5C) but not in their absence (Fig. 5B). These data indicate that IL-6 promoter sequences upstream of −100, and thus upstream of the NF-κB site, are involved in the inhibition of Rta-mediated activation by the NF-κB subunit Rel A.

Since Rel A inhibited the ability of Rta to transactivate the IL-6 promoter only when sequences upstream of the

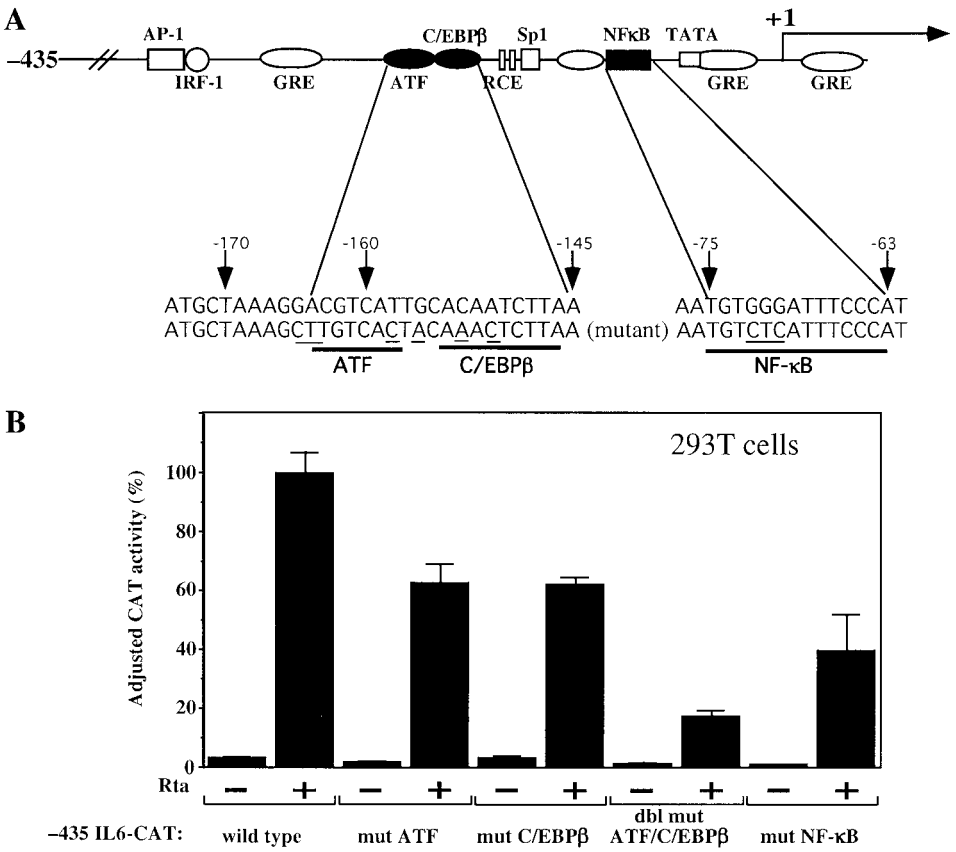

FIG. 4. Rta induces −435 IL-6 CAT reporters despite mutations in the NF-κB site and other consensus elements. (A) Diagram of the wild-type and mutant −435 IL6-CAT constructs: The specific nucleotides that were mutated are underlined. (B) Transient reporter assays were performed in 293T cells with the indicated wild-type and mutant IL6-CAT constructs in the absence and presence of transfected Rta. The level of CAT activity resulting from Rta using the wild-type −435 IL6-CAT was defined as 100%.

NF-κB site were present, this raised the possibility that the inhibition was not a consequence of the interaction of Rel A with the NF-κB site. To test this possibility, responsiveness was assessed using −435 IL-6 CAT with a mutant NF-κB-binding site. Rta effectively induced CAT activity in the absence of Rel A, whereas Rel A was unable to induce reporter activity (Fig. 5D). Cotransfection of Rel A with Rta inhibited the ability of Rta to induce reporter activity with the mutant NF-κB site in −435 IL-6 CAT. Thus, the inhibition of Rta-mediated induction of the IL-6 promoter by Rel A was not dependent on interaction of Rel A with the NF-κB site of the IL-6 promoter.

Rta and the NF-κB subunit Rel A both activate IL6-CAT in HeLa cells through mechanisms involving multiple *cis* elements

HeLa cells were used to compare Rel A and Rta induction of wild-type and mutant −435 IL6-CAT constructs since transactivation of −435 IL-6 CAT by Rel A could not be detected in 293T cells. Transfection of a Rel A expression vector activated −435 IL6-CAT to levels about 50% that of Rta under the same conditions (Fig. 6). Dual mutation of the ATF and 5′C/EBPβ sites eliminated responsiveness to Rel A, whereas the mutant reporter responded to Rta with about 20% of the activity seen with the wild-type −435 IL-6 CAT reporter. As expected, mutation of the NF-κB site eliminated responsiveness to Rel A, whereas responsiveness to Rta was maintained at about 60% of the level seen with the wild-type −435 IL-6 CAT reporter. Thus, promoter elements that were essential for responsiveness to Rel A were not essential for responsiveness to Rta.

Rta induces secreted IL-6 protein in 293T cells

To determine whether Rta induces secretion of cellular IL-6 protein, an Rta expression construct was transfected into 293T cells, and levels of IL-6 in the medium were assayed at 72 h posttransfection (Fig. 7). Rta induced a dose-dependent increase in secreted IL-6 protein which reached levels that were fourfold higher than in medium from untransfected cells or cells transfected with empty vector. Transfection of a Rel A expression construct induced a twofold increase in secreted cellular IL-6. Thus, Rta induced secretion of IL-6 protein, which was consistent with Rta-dependent IL-6 promoter activation in 293T cells.

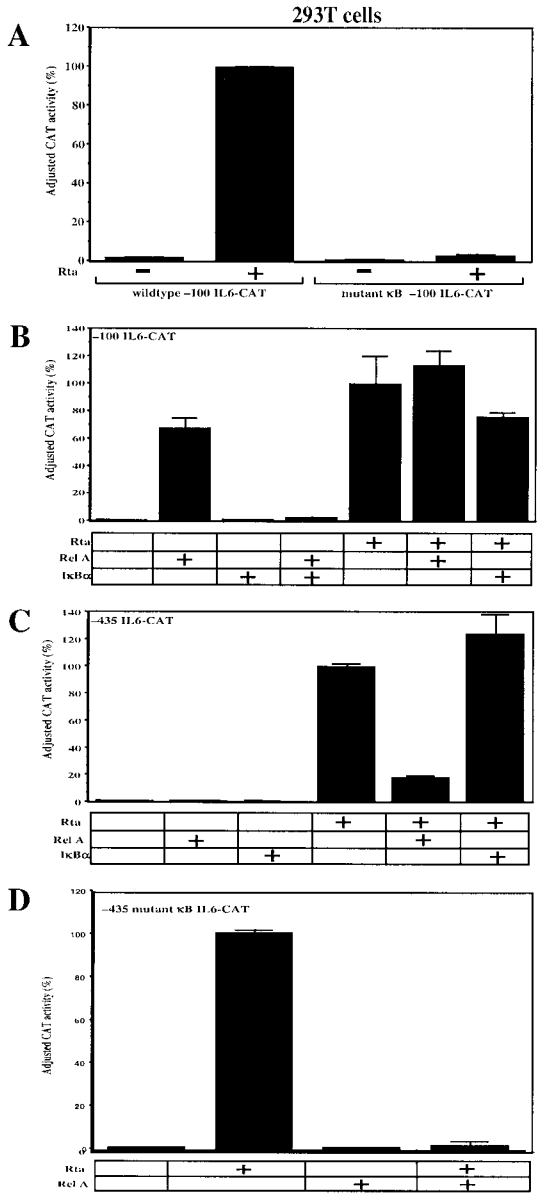

FIG. 5. Rel A inhibits Rta-mediated transactivation of the IL-6 promoter when elements upstream of the κB site are present. (A) 293T cells were transfected with either wild-type −100 IL6-CAT or mutant κB-100 IL6-CAT with or without cotransfected Rta. The level of CAT activity resulting from Rta using the wild-type −100 IL6-CAT was defined as 100%. (B) 293T cells were transfected with wild-type −100 IL6-CAT and with expression vectors for Rta, Rel A, and/or IκBα, as indicated. The level of CAT activity resulting from Rta was defined as 100%. (C) 293T cells were transfected with −435 IL6-CAT and with expression vectors for Rta, Rel A, and/or IκBα, as indicated. The level of CAT activity resulting from Rta was defined as 100%. (D) 293T cells were transfected with −435 IL6-CAT that contained a mutated NF-κB site, and cells were cotransfected with expression vectors for Rta and Rel A as indicated. The level of CAT activity resulting from Rta was defined as 100%.

DISCUSSION

The data presented here broaden our understanding of the possible consequences of Rta expression. We demonstrate that Rta can profoundly affect the function of several cellular promoters in the absence of other HHV-8 factors. Furthermore, the Rel A subunit of NF-κB can inhibit Rta-mediated transactivation of some, but not all, promoters. This suggests that factors that activate NF-κB could suppress the ability of Rta to induce some cellular and viral genes, thereby modulating the consequences of lytic reactivation.

The ability of Rta to transactivate some NF-κB responsive promoters is not likely to be through activation of NF-κB by Rta. We found that the responsiveness of PRDII$_4$-CAT was dependent on sequences that are responsive to NF-κB, yet the induction by Rta was not inhibited by IκBα and thus was distinct from the induction by Rel A. The NF-κB family of transcription factors includes factors in addition to Rel A that can regulate transcription, yet all known transactivating forms of NF-κB are inhibited by IκBα (Ghosh *et al.,* 1998). Furthermore, Rel A inhibited the ability of Rta to transactivate several reporters, including the IL-6 promoter. Such an inhibition would not have occurred if Rta were inducing these promoters through activation of NF-κB.

The transactivation of IL-6 by Rta differs considerably from the transactivation by many other characterized activators. IL-6 is induced in response to a variety of different factors, including IL-1β, TNFα, lipopolysaccharide, and double-stranded RNA (Akira *et al.,* 1992; Akira and Kishimoto, 1997; Dendorfer *et al.,* 1994; Harcourt and Offermann, 2001; Hu *et al.,* 2000; Ray *et al.,* 1989). The ATF/CREB and the C/EBPβ-binding sites are found within two overlapping regions that confer responsiveness to a variety of stimuli, including forskolin, TPA, IL-1β, TNFα, and LPS (Akira *et al.,* 1992; Dendorfer *et al.,* 1994; Harcourt and Offermann, 2000; Hu *et al.,* 2000; Vanden Berghe *et al.,* 2000). These regions are referred to as "multiple cytokine and second messenger response elements (MREs)" and are located between −173 and −145 (Dendorfer *et al.,* 1994; Ray *et al.,* 1990). Our data demonstrate that this region is not required for responsiveness to Rta since the −130 IL-6 CAT construct that lacks this region is as responsive to Rta as larger constructs that contain this region. Furthermore, the NF-κB site that is located at −73 to −63 has been shown to be critical for responsiveness to a variety of stimuli, including IL-1β, TNFα, and dsRNA (Harcourt and Offermann, 2000), yet it is not essential for responsiveness to Rta if upstream sequences are present. For example, the NF-κB site was not required for Rta responsiveness in the −435 IL-6 CAT construct, whereas it was required for Rta responsiveness using −100 IL-6 CAT.

Our studies demonstrate that some promoter constructs lacking the ATF and C/EBPβ-binding sites were as responsive to Rta as constructs containing these sites, yet these sites appeared to contribute to Rta responsiveness when other upstream elements were present. This was shown by dual mutation of the ATF and

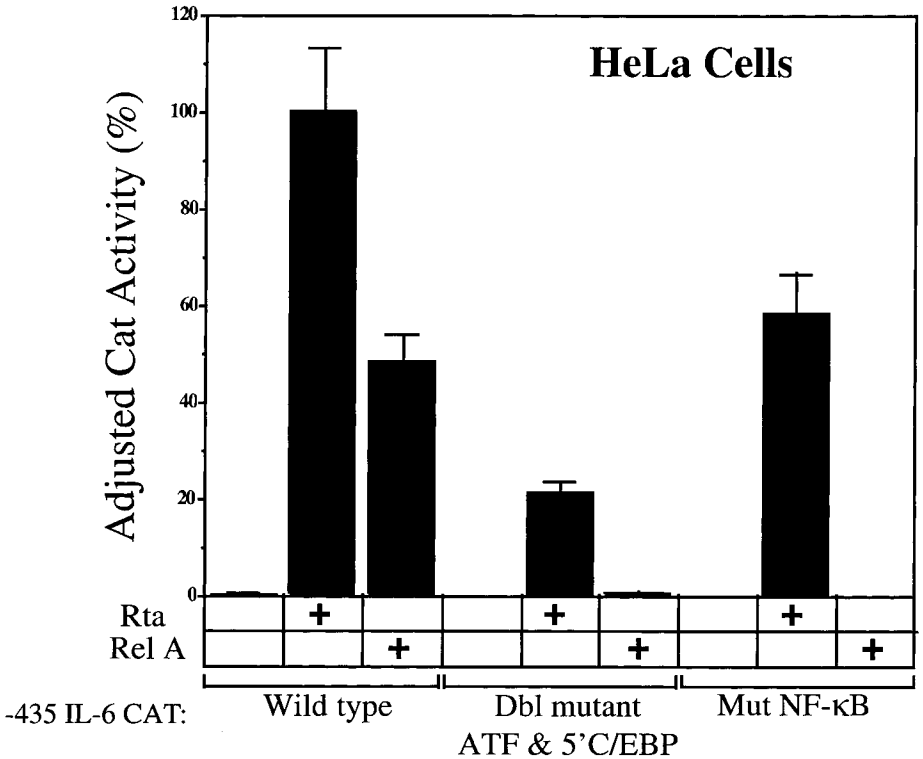

FIG. 6. Mutations in −435 IL6-CAT fully block transactivation by Rel A but not by Rta in HeLa cells. Reporter assays were done in HeLa cells using wild-type or mutant −435 IL6-CAT constructs. The transfections were done using the same procedure as described for the 293T cells with standardization to β-gal as described in Fig. 1. The level of CAT activity resulting from Rta using the wild-type −435 IL6-CAT was defined as 100%.

C/EBPβ sites in the −435 IL6-CAT constructs, where responsiveness to Rta was reduced by 80%. These same mutations completely eliminated responsiveness to Rel A. We have previously shown that HeLa cells transfected with the −435 IL-6 CAT construct containing dual mutation of the ATF and C/EBPβ sites were unresponsive to either IL-1β or to double-stranded RNA, whereas high levels of promoter activity occurred using wild-type −435 IL-6 CAT (Harcourt and Offermann, 2000). The greater importance of these sites in the Rta responsiveness with the −435 IL6 CAT construct than in deletion constructs suggests that their role is dependent on the context of *cis* elements and interactions with other proteins.

The mechanism by which Rta induces transcription of the $PRDII_4$-CAT and IL6-CAT reporters is currently not known. Rta affects gene expression through direct interactions with DNA (Lukac *et al.,* 2001; Song *et al.,* 2001), interactions with cellular transcription factors (Sakakibara *et al.,* 2001), interactions with coactivator proteins (Gwack *et al.,* 2001a,b), and alterations of signal transduction cascades (Gwack *et al.,* 2002). Neither $PRDII_4$-CAT nor IL6-CAT contains consensus elements that resemble known elements that directly bind Rta, including a 31-bp sequence identified in the PAN promoter (Song *et al.,* 2001) and a distinct 12-bp palindromic sequence that is found in the promoters for ORF 57 and k-bZIP (Lukac *et al.,* 2001). While these consensus elements confer Rta responsiveness to heterologous promoters, not all Rta-responsive genes contain these elements. These consensus elements directly bind Rta expressed from a bacterial or baculovirus system, yet they do not bind Rta from nuclear extracts of TPA-induced BCBL-1 cells (Lukac *et al.,* 2001; Song *et al.,* 2001). This suggests that Rta in nuclear extracts does not maintain the ability to directly bind to its consensus elements, thereby complicating the study of interactions between Rta and DNA. Rta has been shown to cooperate with Oct-1 in activating a heterologous promoter containing the Oct-1-binding site (Sakakibara *et al.,* 2001), but neither $PRDII_4$-CAT nor IL6-CAT contain Oct-1 sites. AP1 sites have been shown to contribute to Rta responsiveness in the vIRF-1 promoter through unknown mechanisms (Chen *et al.,* 2000), but our deletion analysis demonstrated that the AP1 site of the IL-6 promoter was not critical for Rta responsiveness. Rta was recently reported to physically associate with Stat 3 and induce dimerization independent of phosphorylation, leading to a transcriptionally active form of Stat 3 (Gwack *et al.,* 2002). One would predict that if a comparable mechanism were involved in the Rta-mediated transcription of IL-6-CAT, then a well-defined consensus element would be required for Rta responsiveness. We did not find evidence of a critical element required for Rta induction of the IL-6 promoter. Our data demonstrated that multiple combinations of elements in the IL-6 promoter contributed to Rta responsiveness. The binding of the transcriptional coactivator CBP by Rta has

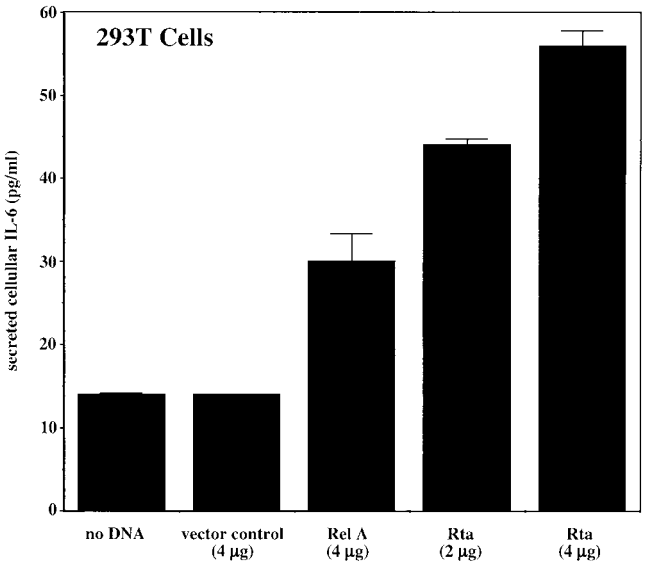

FIG. 7. Rta induces secretion of IL-6 protein in 293T cells. 293T cells were transfected with the indicated amounts of pcDNA-FLAG-ORF 50 (Rta) or pCMV-p65 (Rel A), equalized to 4 μg with pcDNA3.1+. Media was removed 72 h after transfection and assayed for human IL-6 by chemiluminescent ELISA using a standard curve with known concentrations of recombinant IL-6. This representative graph depicts the average secreted cellular IL-6 pg/ml and standard deviation of duplicate samples.

been shown to enhance Rta-mediated transactivation (Gwack *et al.,* 2001a). Transcription factors that bind CBP could either enhance or repress Rta-dependent transcription, depending on whether the protein becomes part of the Rta complex or whether it competes with Rta for the CBP. For example, the CBP-binding proteins c-*jun* and adenovirus E1A had opposite effects on Rta-mediated transcription, with c-*jun* enhancing Rta-mediated transactivation and E1A repressing Rta-mediated transactivation (Gwack *et al.,* 2001a). Two distinct regions of CBP interact with various cellular proteins, with both sites capable of binding to Rta. In addition, domains within both the amino and the carboxyl-terminal regions of Rta can bind to CBP. If the IL-6 promoter was bound by a protein that interacts with one of the CBP domains, then Rta might interact with the other protein-binding domain of CBP. This would allow both CBP and Rta to assemble at the IL-6 promoter in complexes that include cellular DNA-binding proteins. Whether this or other mechanisms are responsible for the observed responses remains to be determined.

The multiple mechanisms by which Rta affects gene expression is reminiscent of the processes affected by its homolog in Epstein–Barr virus, the BRLF1 gene product Rta (Lukac *et al.,* 1998; Sun *et al.,* 1998). EBV-encoded Rta directly binds a GC-rich DNA motif (Gruffat and Sergeant, 1994) but also physically interacts with the retinoblastoma protein (Rb) (Zacny *et al.,* 1998), thereby activating the transcription factors E2F (Swenson *et al.,* 1999). Additionally, EBV-encoded Rta activates the p38 and c-*Jun* N-terminal kinases, resulting in the phosphorylation and activation of the cellular transcription factor ATF2 (Adamson *et al.,* 2000).

The mechanism by which Rel A inhibits Rta-mediated transactivation is not known. The ability of Rel A to inhibit Rta-mediated transactivation was dependent on sequences upstream of the NF-κB site. The −435 IL6-CAT was responsive to Rta but not to Rel A, yet in combination, Rel A blocked Rta induction of this construct. The inhibition of Rta-mediated transaction was not a consequence of competition for binding to the NF-κB site since the inhibition persisted when the NF-κB site in the −435 IL6-CAT construct was mutated. One possibility is that Rel A sequestered CBP, making it unavailable for Rta-mediated transactivation. CBP is physiologically maintained at a limiting concentration in a variety of cells (Kamei *et al.,* 1996), and some CBP-binding proteins such as adenovirus E1A have been shown to inhibit Rta-mediated transactivation. NF-κB requires CBP for activity (Hottiger and Nabel, 2000; Sheppard *et al.,* 1999), and sequestration of CBP by NF-κB would not require a functional NF-κB site to inhibit Rta-mediated transcription. When the IL-6 promoter sequences upstream of the NF-κB site were deleted, there was no longer clear evidence of inhibition of Rta-mediated induction by Rel A. Since the truncated promoter was also responsive to Rel A, it is possible that the observed activity resulted from Rel A. Alternatively, the inhibition of Rta transactivation by Rel A required sequences that were lacking in the −100 IL6-CAT construct.

The ability of Rel A to inhibit Rta-mediated transcription extends to viral promoters. For example, we found that Rel A inhibited transactivation of the vIRF-1 promoter by Rta (F. Roan, N. Inoue, and M. K. Offermann, unpublished data), and other investigators have found that Rel A inhibits Rta-dependent induction of the HHV-8 PAN promoter as well as its own promoter (H. J. Brown and R. Sun, personal communication).

Cellular gene expression resulting from Rta might contribute to the pathogenesis of diseases associated with HHV-8. We have shown that Rta is sufficient to induce IL-6 secretion. IL-6 is overexpressed in Kaposi's sarcoma, primary effusion lymphoma, and Castleman's disease and can act as an autocrine or paracrine growth factor (Aoki *et al.,* 2001; Dourado *et al.,* 1997; Drexler *et al.,* 1999; Foussat *et al.,* 1999; Miles *et al.,* 1990; Oksenhendler *et al.,* 2000). Considerable lytic replication is present in Castleman's disease, whereas HHV-8 is primarily latent in both KS and PEL (Dupin *et al.,* 1999; Judde *et al.,* 2000; Sturzl *et al.,* 1997). Cytokines such as TNFα and IL-1β that induce IL-6 expression are present in many KS lesions (Oxholm *et al.,* 1989), indicating that factors independent of Rta are likely to contribute to the high levels of IL-6 that occur. Nonetheless, the ability of Rta to induce cellular genes might be of importance in some of the changes that result during lytic replication.

The high levels of TNFα and IL-1β that are expressed in KS lesions would be expected to activate the transcription factor NF-κB. It is intriguing to speculate whether activated NF-κB could suppress the response to Rta in KS lesions, thereby helping to maintain HHV-8 in the latent state.

MATERIALS AND METHODS

Cell culture

293T and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Herdon, VA) and MEM (Mediatech), respectively, supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 5 mM L-glutamine (Gibco-BRL, Rockville, MD), and 100 U/ml penicillin-streptomycin (Gibco-BRL).

Plasmid constructs

pCMV-p65, pCMV-IκBα, and HIV(κB)$_4$-CAT were kindly provided by Charles Kunsch and have been described previously (Kunsch *et al.,* 1992; Ruben *et al.,* 1992). The reporter constructs −128 β-CAT, PRDI$_4$-CAT, PRDII$_4$-CAT, and PRDIII$_6$-CAT were provided by Tom Maniatis and also have been described previously (Fan and Maniatis, 1989; Keller and Maniatis, 1988). The deletion constructs of the IL-6 promoter and the −435 IL6-CAT constructs containing mutations in the ATF, C/EBPβ, and/or NF-κB sites were described previously (Harcourt and Offermann, 2000). The construct −100 mutκB IL6-CAT was generated in two steps. First, the appropriate region from −435 mutκB IL6-CAT was PCR amplified and cloned into pGEM-T easy (Promega, Madison, WI) to generate −100 mutκB-TE. Next the *Sac*I-*Nco*I fragment from −100 mutκB-TE was cloned into the corresponding sites of pCAT3-Basic. The construct was confirmed by sequencing (Emory DNA Sequencing Core Facility).

To generate an expression vector for Rta, exon 2 of Rta was PCR amplified from BC-1 DNA using the primers 5′-TCCCCGGGTAAGAAGCTTCGGCGGTCCTGT-3′ and 5′-CCGAATTCTGTAGGTTAACTCCACTTTGCACC-3′. This PCR product was digested with *Sma*I and *Eco*RI and cloned into pBluescript II KS+ (Stratagene, La Jolla, CA) to generate the plasmid pBS-ORF50B. A small DNA fragment containing a *Bam*HI site, a Kozak consensus sequence, an amino-terminal FLAG tag, and exon 1 of Rta, which is located upstream of ORF 49, was generated by annealing two oligonucleotides, 5′-GATCCACCATGGATTACAAGGATGACGACGATAAGATGGCGCAAGATGACAA-3′ and 5′-TTGTCATCTTGCGCCATCTTATCGTCGTCATCCTTGTAATCCATGGTG-3′. The complete FLAG-tagged Rta coding region was generated by cloning the exon 1 fragment between the *Bam*HI and *Sma*I sites of pBS-ORF50B. The Rta-coding region was then excised with *Not*I and *Eco*RI and cloned into pCMV-Script (Stratagene). pcDNA-FLAG-ORF 50 was generated by subcloning the tagged Rta coding region into the *Xba*I and *Eco*RI sites of pcDNA3.1+ (Promega). A nontagged Rta expression vector, pCMV-FLc50, and an expression vector for a Rta truncation lacking the transactivation domain (pCMV-myc-nuc-50ΔSTAD) were generously provided by Don Ganem and were previously described (Lukac *et al.,* 1999).

Transfections and reporter assays

293T cells were split to six-well plates approximately 24 h prior to transfection and transfected at 50 to 70% confluency using FuGENE 6 (Roche, Indianapolis, IN) according to the manufacturer's protocol. Transfections for enzyme-linked immunosorbent assays (ELISAs) consisted of 2 or 4 μg of pcDNA-FLAG-ORF 50 or pCMV-p65, equalized to 4 μg with pcDNA3.1+. DNA mixtures for transfections for reporter assays contained 0.9 μg of CAT reporter plasmid, and 0.25 or 0.75 μg of expression vectors for transcription factors (e.g., Rta or Rel A) or inhibitors (e.g., IκBα), respectively. In transfections for reporter assays, 0.1 μg of pEF-LacZ, a plasmid expressing β-galactosidase under the control of the eukaryotic elongation factor-1α promoter, was added and used as an internal control for transfection efficiencies, and total amounts of DNA were equalized to 2 μg using pcDNA 3.1+. Cells were harvested 24–72 h after transfection and assayed for CAT and β-galactosidase activity. Crude lysates for reporter assays were obtained by lysis of the cells using reporter lysis buffer (Promega) according to the manufacturer's instructions.

β-galactosidase activity was measured on a LUMIstar Galaxy luminometer (BMG Biotechnologies, Durham, NC) using the Galacto-Light Plus chemiluminescent reporter gene assay system, according to the manufacturer's instructions (Applied Biosystems, Bedford, MA). β-galactosidase activity in the lysates was determined based on a standard curve generated using recombinant β-galactosidase (Promega). Chloramphenicol acetyl transferase (CAT) activity was determined by phase extraction of butyrylated [^{14}C]chloramphenicol (Amersham-Pharmacia, Piscataway, NJ) followed by scintillation counting as previously described (Kingston and Sheen, 1997). Adjusted CAT activity was then obtained by normalizing to relative levels of β-galactosidase to correct for differences in transfection efficiencies. Results were obtained as mean ± standard deviation from experiments performed in triplicate.

IL-6 ELISA assays

Medium was taken from transfected 293T cells and assayed for human interleukin-6 with the ImmuLux human IL-6 chemiluminescent ELISA, using monoclonal antibodies, according to the manufacturer's instructions (Pierce Endogen, Rockford, IL).

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